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Recombinant *Mycobacterium bovis* BCG producing the circumsporozoite protein of *Plasmodium falciparum* FCC-1/HN strain induces strong immune responses in BALB/c mice

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Abstract

The current vaccine against tuberculosis, *Mycobacterium bovis* strain bacillue Calmette–Guerin (BCG), offers potential advantages as a live, innately immunogenic vaccine vehicle for expression and delivery of protective recombinant antigens. Malaria is one of the severest parasitic diseases in humans especially in the developing world. No efficacious vaccine is currently available. However, circumsporozoite protein (CSP) is a malaria vaccine candidate currently undergoing clinical trials. We analyzed the immune response to recombinant BCG (rBCG) vaccine expressing *Plasmodium falciparum* CSP (BCG-CSP) under the control of heat shock protein 70 promoter in BALB/c mice. The lymphocytes proliferative response to *P. falciparum* soluble antigen was significantly higher than those in the groups of BCG and normal saline, and the production of cytokines (IFN-γ and IL-2) in response to malaria antigen was significantly higher in rBCG and BCG groups than control group of normal saline. A specific IgG antibody response against *P. falciparum* antigen of CSP was also characterized. The booster injection could enhance the production of cytokine, proliferation responses of spleen lymphocytes and the antibodies titer of BCG-CSP. The results in the study demonstrated that rBCG vaccine producing CSP is an appropriate vaccine for further evaluation in non-human primates.

Keywords: *Plasmodium falciparum*; Circumsporozoite protein; BCG; Immune response; Recombinant

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1. Introduction

Malaria, a disease caused by protozoan parasites of the genus *Plasmodium*, is one the most dangerous infectious diseases affecting human populations. Approximately 300–500 million people are infected annually, and 1.5–2.7 million lives are lost to malaria each year, with most deaths occurring among children in Sub-Sahara Africa [1]. Of the four species that cause malaria in humans, *Plasmodium (P.) falciparum* is the greatest cause of morbidity and mortality. The resistance of the malaria parasite to drugs and insecticides has resulted in a resurgence of malaria in many parts of the world. The main hope of controlling the disease is the development of safe and effective vaccines [1,2].

It has been demonstrated that, under experimental conditions, humans [3] and animals [4] could be protected by immunization with the bites of irradiated infected mosquitoes or by direct injection of radiation-attenuated sporozoites. This protection is mediated in part by antibodies, some of which are directed against the repeat region of the circumsporozoite protein (CSP) which covers the sporozoite surface [5]. On the other hand, animal experiments have indicated that *P. falciparum* sporozoites induce CSP-specific $CD8^+$ cytotoxic T lymphocytes (CTL) [6] and that T cells alone are sufficient for sporozoite-induced immunity in mice [7]. More recently, cloned CTL cell lines directed against the *Plasmodium berghei* CSP have been shown to passively transfer protection against challenge [8]. These results strongly suggest that the use of live recombinant vehicles might adequately present the antigen to the immune system. In this context, Kumar et al. [6] and Sadoff et al. [9] reported the use of vaccinia and attenuated *Salmonella*, respectively expressing the full length *P. berghei* CSP antigen. Aggarwal et al. [10] also demonstrated that orally administered *Salmonella*-CSP recombinants induced protective CSP-specific CTLs.

Mycobacterium (M.) bovis BCG is another attractive candidate for the development of live recombinant vaccines. Indeed, BCG has already been used widely over the years without significant adverse effects. It has been recommended by the World Health Organization to be given at birth and requires only a single immunization to protect efficiently against tuberculosis. It is the most heat stable of live vaccines, stimulates cell-mediated immunity in animals and humans and is particularly cheap and in terms of production and use [11]. The use of *Mycobacterium smegmatis* and *M. bovis* BCG as hosts for the expression of foreign DNA has already been documented [12–16] and several tools are now available to construct efficient expression vectors for *Mycobacteria*.

Huangfu et al. [17], for instance, described the *Mycobacterium–Escherichia coli* shuttle vector pBCG2000, then the 150 bp length promoter of human *M. tuberculosis* heat shock protein (HSP 70) was introduced into it to construct the pBCG2100 [18]. We took advantage of these genetic elements and combined them to construct an expressing vector wherein the DNA coding for CSP of *P. falciparum* was placed under the control of HSP70 promoter, namely pBCG /CSP [19].

The data presented here show that the recombinant shuttle plasmids pBCG /CSP-transformed *M. bovis* BCG could induce a strong humoral and cellular immune response in BALB /c mice. These results open the way to other animal and human testing of a new malaria vaccine.

2. Material and methods

2.1. Culture of recombinant BCG and BCG

The rBCG containing recombinant shuttle plasmid pBCG /CSP (BCG-CSP) [19], constructed by our laboratory, and BCG (Denmark strain, The Beijing Institute of Biological Products, PR China) was cultivated in liquid Middlebrook 7H10 medium (Difico Laboratories, Detroit, MI, USA) supplemented with 10% Middlebrook 7H10 Enrichment ADC (Albumin–dextrose-catalase complex) (Difico Laboratories) and 0.05% Tween 80 (M-ADC-TW broth) and kanamycin (10 $\mu g\ ml^{-1}$) with shaking at 37 °C. The culture CFU was estimated by determination A600, with 0.1 optical density (OD) equal to $10^8\ ml^{-1}$ and stored as frozen aliquots for immunization.

2.2. Animals and immunization procedures

The specific pathogen-free BALB/c mice were obtained from Laboratory Animals Center of the Chinese Academy of Medical Science (Beijing, PR China). A single dose, approximately 1×10^7 CFU bacilli suspended in PBS, of non-transformed BCG or BCG-CSP were administered to groups of six 6-week-old BALB/c mice by subcutaneous route. Four weeks later, the mice were boosted with the same dose as the first immunization. An additional group was composed of non-immunized mice. Sera from each group of mice were collected before and at 4 weeks after boosting. The antibody response against CSP was analyzed by enzyme-linked immunosorbent assay (ELISA).

2.3. Splenocyte preparation

Spleens from immunized and control mice were homogenized, and erythrocytes were lyzed with lysis buffer (Sigma). The cells were then washed with complete RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 unit ml^{-1} penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin and adjusted to the proper concentration.

2.4. Preparation of Plasmodium soluble antigen (PSA)

Tachyzoites of *Plasmodium falciparum* were obtained from the culture and treated with erythrocyte lysis buffer, then the parasites were washed, resuspended in phosphate-buffered saline (PBS), and sonicated in an Ultrasonic disintegrator (MSE, Leicester, UK). The protein concentration of *Plasmodium* lysate (PSA) was determined by using the Bio-Rad DC protein assay and albumin to generate a stand curve.

2.5. ELISA for antibody assay

Sera samples were collected at time points before vaccination and before and after boosting and pooled for each group to monitor antibody responses by ELISA, as described elsewhere. Each well of the microtiter plates was coated with 50 μl of PSA at the concentration of 30 $\mu g\ ml^{-1}$ in phosphate-buffered saline (PBS) for 2.5 h at 37 °C or overnight at 4 °C. The PSA solutions were removed, and the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T20) shaking on the automatic ELISA washer. Two-fold serial dilution of serum starting at 1:200 was performed in PBS-T20 containing 0.05% (w/v) gelatin, and 50 μl of each sample was added to individual wells of the Ag-coated plates, and incubation was continued overnight at 4 °C or 2.5 h at 37 °C. The plates were then washed five times with PBS-T20 and incubated for 30 min at 37 °C with 100 μl peroxidase-conjugated goat anti-mouse IgG (The Shanghai Institute of Biological Products, PR China). After washing with PBS-T20 four times, Color was developed with 100 μl of *O*-phenylenylene diamine (0.4 $\mu g\ ml^{-1}$) (Sigma) solution in 80 mM citrate-phosphate (pH 5.0) and measured by absorbance at 492 nm optical density on an ELISA reader. Endpoint titers were defined as the highest dilution at which the A492 values were twice the values for pre-immune sera diluted 1:200 in PBS-T20. Individual titers were expressed as the log10 of the endpoint titer. Titers ($\pm$standard errors, S.E.) were calculated as arithmetic mean of the $\log_{10}$ titer.

2.6. Measure of P. falciparum CSP specific lymphocytes proliferation

To measure CSP specific cellular immune responses, spleen lymphocytes were prepared at time points before vaccination and before and after boosting. For the lymphocyte proliferation assay, 1×10^5 purified lymphocytes per well were incubated in complete RPMI 1640 in 96-well round-bottom plates with 10 $\mu g\ ml^{-1}$ PSA or *Toxoplasma gondii* cell lysate as an antigen control for 5 days ending with an 18 h pulse of [^{3}H]thymidine at 1 μCi/well. The plates were harvested onto fiberglass filters and counted by liquid scintillation. The stimulation index (SI) was calculated as the ratio of counts per minute in stimulated wells divided by counts in antigen control wells. An index of ≥ 2.0 was considered positive.

2.7. Cell phenotyping

Monoclonal antibodies (mAbs) used to quantitate the mice lymphocyte subsets were purchased from Ebioscience (German). The mAb used were specific for the following lymphocyte subsets: FITC-labeled anti-mouse CD3e (Hamster IgG, 145-2c11, total T cells), PE-labeled anti-mouse CD4 (L3T4) (Hamster IgG, 145-2c11, helper T cells), and PE-labeled anti-mouse CD8a (Lyt-2) (Rat IgG2a, Kappa, 53-6.7, cytotoxic T cells). Purified spleen lymphocytes were stained with the mAbs listed above. Normal splenocytes were stained as the control and the isotype antibodies FITC-labeled Hamster IgG and PE-labeled Rat IgG2a kappa were used for staining as controls to CD3, CD4 and CD8 respectively. Double color flow cytometry was used to determine the changes in lymphocyte subsets.

2.8. Generation and assay for interferon-gamma (IFN-γ) and interleukin 12 (IL-12)

An ELISA method for cytokines assay was employed to quantify the level of cytokines secreted in the supernatant of lymphocyte cultures. Purified spleen cells were adjusted to a concentration of 5×10^6 ml^{-1} and grown in flat-bottomed microwell plates (Nunc) in complete RMPI-1640 medium. The PSA was added the cultures at 10 μg ml^{-1}. Each test was performed in triplicate. Cells were incubated at 37 °C in humidified air with 5% CO_2. After 24 h culture, the cell-free culture supernatants were harvested for the determination of IFN-γ and IL-2 production by quantitative sandwich enzyme immunoassay Kits (Diaclone, France) according to the instruction of the manufacturer. Each experiment was repeated at least three times, and the results of one representative experiment are shown.

2.9. Statistical analysis

The statistical difference was tested by Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Systemic antibody response of BALB/c mice immunized with BCG-CSP

To evaluate the capacity of BCG-CSP to stimulate humoral immune responses in mice, we, therefore, immunized BALB/c mice and analyzed their anti-CSP antibody responses. As shown in Table 1, BALB/c mice immunized with 1×10^7 CFU of BCG-CSP developed a significant antibody response against CSP which further increased following a booster injection with 1×10^7 CFU of BCG-CSP at day 29.

3.2. Proliferative responses

After subcutaneous immunization with wild-type BCG or BCG-CSP, in vitro proliferative responses to malaria antigen of splenocytes were analyzed. Specific proliferative responses were induced, as shown in Table 2, splenocytes isolated from mice immunized with BCG-CSP proliferated vigorously in response to malaria soluble antigens, and the proliferation response was accelerated following booster administration. The spleen lymphocytes of non-transformed BCG group also proliferated to a certain degree due to the non-specific stimulation of BCG, but there was remarkable difference between the BCG and BCG-CSP groups. This indicated that the proliferation of spleen lymphocytes was specific to CSP.

Table 1
Anti-CSP endpoint titers of BALB/c mice immunized with BCG-CSP

Immunogen	Mean anti-CSP endpoint titer[a]		
	4 week	8 week	8 week[b]
BCG control	200 ± 0	400 ± 0	200 ± 0
BCG-CSP	976 ± 134[c]	2685 ± 432[c]	6400 ± 0[c,d]

[a] ELISA to *P. falciparum* soluble antigen.
[b] Booster group.
[c] $P < 0.05$, as compared with BCG control group.
[d] $P < 0.05$, as compared with BCG-CSP immunized group without booster injection.

Table 2
Proliferative response induced by *P. falciparum* soluble antigen in spleen lymphocyte from BCG-CSP immunized BALB/c mice

Immunogen	Stimulation index[a]		
	4 week	8 week	8 week[b]
Normal saline	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.3
BCG control	2.2 ± 0.4	2.1 ± 0.2	2.3 ± 0.2
BCG-CSP	3.9 ± 0.6[c]	4.3 ± 0.5[c]	7.3 ± 0.7[c,d]

[a] Proliferative response in the responding cultures(values were expressed as mean ± S.E.).
[b] Booster group.
[c] $P < 0.05$, as compared with BCG and normal saline control group.
[d] $P < 0.05$, as compared with BCG-CSP immunized group without booster injection.

3.3. Lymphocyte subsets

The changes of the percentages of CD3 /CD4$^+$ T cells, CD3/CD8$^+$ T cells and the ratio of CD4$^+$/CD8$^+$ are illustrated in Table 3. The immunization of BCG-CSP and the booster injection did not significantly influence the percentages of any of the CD3 /CD4$^+$ T lymphocytes, but the percentages of CD3 /CD8$^+$ T lymphocytes significantly increased, and the ratio of CD4$^+$/CD8$^+$ was reduced and reversed. There was a remarkable difference between the normal saline control and the BCG or BCG-CSP immunized group, but there was no remarkable difference between the BCG and BCG-CSP groups.

3.4. Cytokine production

At 4 and 8 weeks after subcutaneous immunization and 4 weeks after the booster injection with wildtype BCG or BCG-CSP, splenocytes were recovered and analyzed for in vitro cytokine production. After incubation, the cytokines production of spleen lymphocytes in response to the malaria antigens was assessed by ELISA, and the results are presented in Table 4. An appreciable amount of IFN-γ and IL-2 were detected in BCG-CSP immunization groups, which further increased following the booster injection. Although the spleen lymphocytes of the non-trans-

Table 3
The kinetic changes of splenic T lymphocyte CD3/CD4$^+$, CD3/CD8$^+$ subsets and its CD4$^+$/CD8$^+$ ratio[a]

Immunogen	4 weeks			8 weeks		
	CD3/CD4$^+$	CD3/CD8$^+$	ratio	CD3/CD4$^+$	CD3/CD8$^+$	ratio
Normal saline	29.7 ± 3.1	15.6 ± 2.4	1.9 ± 0.1	32.3 ± 1.9	17.5 ± 2.9	1.8 ± 0.2
BCG control	30.2 ± 2.6	30.4 ± 1.7[b]	1.0 ± 0.1[b]	32.8 ± 2.3	29.8 ± 1.9[b]	0.9 ± 0.2[b]
BCG-CSP	30.7 ± 2.4	33.9 ± 1.9[b]	0.9 ± 0.1[b]	31.6 ± 2.5	32.1 ± 2.3[b]	1.0 ± 0.1[b]

[a] The results of booster injection group were similar to BCG-CSP group(data not shown) .
[b] $P < 0.05$, as compared with normal saline control group.

Table 4
The production of cytokines in culture supernatant of spleen lymphocyte

Immunogen	4 weeks		8 weeks		8 weeks[b]	
	IFN-γ	IL-2	IFN-γ	IL-2	IFN-γ	IL-2
Normal saline	86	46	78	41	92	53
BCG control	146	89	172	79	178	85
BCG-CSP	987[c]	203[c]	1085[c]	217[c]	1587[c,d]	283[c,d]

[a] The unit of cytokines is pg ml^{-1}.
[b] Booster group.
[c] $P < 0.05$, as compared BCG and normal saline control group.
[d] $P < 0.05$, as compared with non-booster group.

formed BCG group produced cytokines to a certain degree due to the non-specific stimulation of BCG, there was remarkable difference between BCG and BCG-CSP group. It indicated that the cytokines was specific to CSP.

4. Discussion

Vaccines represent a cost-effective approach to control of infectious diseases [20]. The present study demonstrated that recombinant BCG vaccine expressing *P. falciparum* CSP not only could induce antibodies responses to a certain degree, with highest titer of 6400, but also cellular immune responses in BABL/c mice. The immunization of BCG-CSP could induce the production of IFN-γ and IL-2 as representative of Th-1 responses in splenocytes, and enhance the percentage of CD3/CD8 T cells, namely cytotoxic T cells, which will promote the host to kill exo-pathogens, and boost up the proliferation of spleen cells in vitro after the stimulation of malaria antigens. The booster injection could enhance the cellular immune responses to a certain degree.

Significant progress has been made toward the identification of protective antigens for a wide variety of diseases, but basic difficulties central to practical vaccine development still exist. Among the most formidable obstacles is the cost-effective production of immunogenic vaccine components in sufficient quantities for wide use and the delivery of these components in a safe and immunogenic form. One approach taken to overcome vaccine production and delivery problems is the development of live attenuated vaccines that replicate and express protective antigens in vivo. With the development of recombinant DNA methodology has come approaches to modify live attenuated vaccines, (e.g. pox viruses and *Salmonella*) to produce heterologous antigens protective against tuberculosis, the live attenuated BCG vaccine also offers considerable advantages for development as a multi-valent vaccine vehicle for other human pathogens [12]. With the development of genetic vector systems for the slow-growing mycobacteria, it is now a multi-valent live carrier for inducing protective immunity to heterologous antigens [12,14]. Inasmuch as BCG is rapidly ingested by macrophages and grows within the macrophage phagolysosome, rBCG is usually thought of as a vaccine vehicle for eliciting stronger cellular responses than humoral responses.

To date, CSP is the only malaria antigen that, when used as a subunit vaccine, has conferred protection against experimental sporozoite challenge in human volunteers [21–23], and CSP vaccine candidates have shown promise in clinical trials [24]. Therefore, CSP was chosen as candidate antigen for the construction of a recombinant BCG vaccine for malaria.

In conclusion, the study has provided a demonstration of the humoral and cellular immune response elicited by the recombinant BCG vaccine carrying the *P. falciparum* CSP gene and the development of a promising candidate vaccine for the immunoprophylaxis of malaria. Further studies are necessary to determine whether the recombinant BCG vaccine could elicit protective immunity in appropriate animal models.

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